The Enzymic Activity of Trypsin Autolysis Products[1]

LEOPOLD WEIL[2] AND SERGE N. TIMASHEFF

Eastern Regional Research Laboratory,[3] Philadelphia, Pennsylvania 19118

Sedimentation experiments on trypsin and trypsin derivatives have shown that a small residual enzymic activity is present in the degradation products after autolysis of the enzyme.

In recent years a number of reports have been made to the effect that breakdown products of trypsin autolysis retain part of the enzymic activity of the parent molecule (1–7). In their study, Bresler *et al.* (1) have found that, after sedimentation of a trypsin autolyzate, 5–10% of the activity remained in the supernatant solution. When a fresh preparation of trypsin was sedimented, however, a small amount of activity was again found in the supernatant solution. Thus, the low residual activity of the autolyzate could conceivably be due to the presence of undegraded trypsin which had not been sedimented to the bottom of the cell, either because of occlusion on cell walls or by back diffusion. We have re-examined this problem using as controls solutions of trypsin under conditions at which it is not subject to autolytic degradation; such a degradation can be prevented by the presence of calcium ions (8) or by the previous reaction of the enzyme with diisopropylfluorophosphate (DFP). This second treatment could be used in this study since the DFP-treated enzyme is known to retain some hydrolytic activity toward salmine (9).

EXPERIMENTAL PROCEDURE

The trypsin used was a Worthington[4] crystalline preparation. Assays were carried out with *p*-toluenesulfonyl ethyl ester (TSAEE) and salmine (in the presence and absence of DFP) used as substrates, as described previously (9). To prepare the diisopropyl derivative, 20 mg of trypsin, dissolved in 1 ml of water, was mixed with 0.2 ml of 0.05 M DFP and 0.8 ml of 0.2 ionic strength phosphate buffer of pH 8. After standing overnight in the refrigerator a small amount of precipitate was centrifuged off. The derivative was found not to hydrolyze TSAEE, but was still active toward salmine as substrate (9). Sedimentation experiments were carried out in a Spinco model E analytical ultracentrifuge, using a separation cell. All runs were performed at 59,780 rpm and 12°. Most experiments were done in pH 8 phosphate buffer of 0.1 ionic strength; the run in the presence of calcium ions was performed in a pH 4.7 calcium acetate buffer of 0.1 ionic strength.

RESULTS

Figure 1 shows the sedimentation patterns obtained under four sets of conditions. In each case the sedimentation proceeds from right to left; the vertical bar in each picture is the partition which divides the cell into two compartments. The enzymic activities toward salmine, both of the original material and of the two fractions after sedimentation, are summarized in Table I. However, when TSAEE was used as substrate, none of the fractions displayed any activity, except, of course, for the freshly dissolved active trypsin and its sedimented fraction. Comparison of the early sedimentation patterns with those found at the end of the run (8 hours) and simultaneous ex-

[1] Dedicated to Luis F. Leloir on the occasion of his sixtieth birthday.

[2] Deceased December 7, 1964.

[3] Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[4] It is not implied that the USDA recommends the above company or its product to the exclusion of others in the same business.

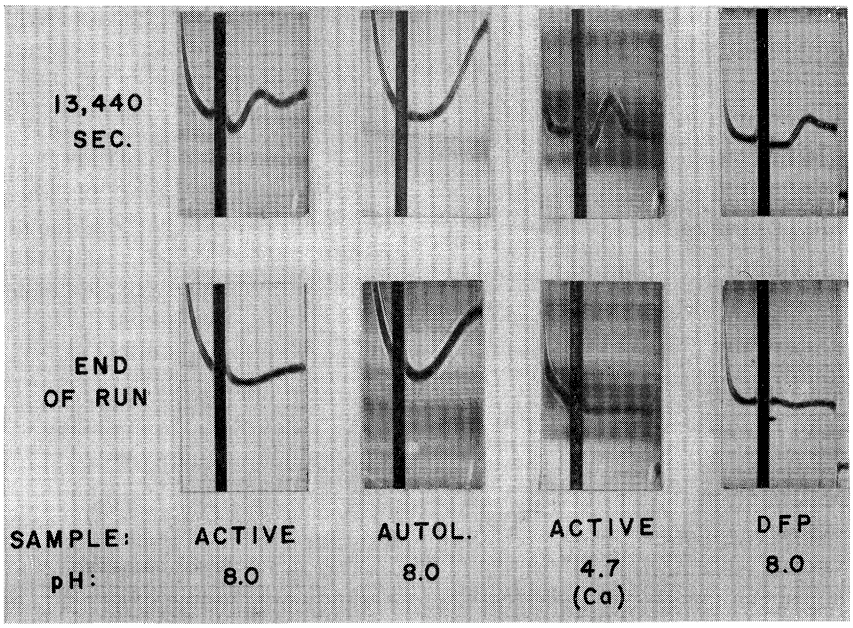

FIG. 1. Sedimentation patterns of various trypsin preparations. For details, see text.

TABLE I

ACTIVITY OF TRYPSIN FRACTIONS

Treatment	Conc. (mg N/ml)			α-NH_2-N liberated by 0.1 ml (mg)[a]		
	Whole	Sed.	Supern.	Whole	Sed.	Supern.
Active, pH 8.0	—	2.15	0.39	—	1.34	0.83
Autolyzed	5.30	5.28	4.63	3.90	2.08	1.40
CaAc, pH 4.7[b]	1.45	3.52	0.27	0.30	0.76	0.04
DFP, Run 1	1.20	1.60	0.38	0.50	0.76	0.03
DFP, Run 2	0.85	1.76	0.41	0.58	1.32	0.06

[a] Salmine used as substrate; 7-hour digestion.
[b] Assayed in the presence of DFP.

amination of the activity data leads to the following observations:

(*1*) In the case of freshly dissolved, active trypsin, the bulk of the enzyme sediments completely during the course of the run, with a small amount of nonsedimenting material remaining in the body of the solution. Both the original material and the sedimented fraction hydrolyze TSAEE; no such activity is detectable in the supernatant material. Prolonged digestion of salmine shows some activity to be present in all fractions.

(*2*) In the case of trypsin autolyzed by standing for 3 days at 30° in pH 8 phosphate buffer, no sedimenting boundary due to intact protein is detectable, but material is distributed throughout the cell. Salmine was digested by all the fractions, but none were active if TSAEE were used as substrate.

(*3*) When active trypsin was centrifuged in calcium acetate buffer at pH 4.7, most of the protein sedimented. Digestion of salmine in the presence of DFP revealed a small amount of activity remaining in the starting material and the sedimentable fraction; no such activity could be detected in the supernatant solution.

(*4*) Sedimentation of the diisopropyl derivative resulted in a pattern similar to case *3*; most of the material sedimented to the bottom of the cell. Both the derivative and its sedimented fraction were active in the

TABLE II

DIALYSIS OF AUTOLYZED TRYPSIN

Conditions	Volume of dialyzate	N in dialyzate (mg/ml)	α-NH_2-N liberated by 1 ml of dialyzate (mg)[a]
2.4 ml autolyzate (7.8 mg N/ml) vs. 0.1 M PO_4, pH 8 (24 hours)	6	1.16	1.11
1 gm cryst. trypsin (50% Mg SO_4) autolyzed with simultaneous dialysis vs. 0.1 M PO_4, pH 8 (24 hours)	194.0	0.31	0.41

[a] Salmine used as substrate; 7-hour digestion.

digestion of salmine, but the supernatant fraction was devoid of activity.

In view of the enzymic activity displayed by the nonsedimenting fraction in the case of fresh and autolyzed trypsins, dialysis experiments were carried out on these two materials. The results are summarized in Table II. In the case of the autolyzate, after 24 hours of dialysis the nitrogen had become evenly distributed on the two sides of the membrane. There was no detectable activity toward TSAEE, but salmine was digested. In the case of the crystalline enzyme, more than half of the nitrogen had dialyzed out after 24 hours, with 35% of the activity toward salmine being present outside of the bag.

DISCUSSION

The above experiments show that autolysis results in the complete loss of tryptic activity toward TSAEE. The sedimentation experiments in the presence of calcium ions or DFP, with the subsequent assay using salmine as substrate in the presence of DFP, show that, when autolysis is absent, no residual activity is left behind in the supernatant solution. This indicates that when activity is found in the supernatant fraction, it is very unlikely that it is due to the presence of nonsedimented intact enzyme molecules. It would seem, thus, that the activity found in the supernatant fraction in all cases resides probably in active degradation products. These conclusions are supported by the results of the dialysis experiments. While it has been reported that trypsin molecules pass through a dialysis membrane (10), the rapid equilibration across the membrane concomitant with lack of activity toward TSAEE suggests preferably that the activity toward salmine resides in the degradation products. Furthermore, the fact that the enzymic activity could be detected only on prolonged digestion of salmine indicates that the tryptic activity remaining in the products of autolysis is quite low; under normal conditions, salmine has been shown to be degraded very rapidly by trypsin (9). Finally, it might of interest to point out that the isolated electrophoretically rapid fraction of trypsin (11) behaves in a manner similar to the unfractionated enzyme.

REFERENCES

1. BRESLER, S. E., GLIKINA, M. V., AND FRENKEL, S. YA., *Doklady Akad. Nauk. USSR* **96,** 565 (1954).
2. NORD, F. F., BIER, M., AND TERMINIELLO, L., *Arch. Biochem. Biophys.* **65,** 120 (1956).
3. HESS, G. P., AND WAINFAN, E., *J. Am. Chem. Soc.* **80,** 501 (1958).
4. BRESLER, S., CHAMPAGNE, M., AND FRENKEL, S., *J. Chim. Phys.* **59,** 180 (1962).
5. WAINFAN, E., AND HESS, G. P., *J. Am. Chem. Soc.* **82,** 2069 (1960).
6. HAKIM, A. A., PETERS, R. L., SAMSA, E. G., AND VAN MELLE, P. J., *Enzymologia* **25,** 134 (1962).
7. IACHAN, A., DOMONT, G. B., DISITZER, L. V., AND PERRONE, J. C., *Nature,* **203,** 43 (1964).
8. NORD, F. F., AND BIER, M., *Biochim. Biophys. Acta* **12,** 56 (1953).
9. WEIL, L., AND TELKA, M., *Arch. Biochem. Biophys.* **71,** 204 (1957)
10. CRAIG, L. C., KING, T. P., AND STRACHER, A., *J. Am. Chem. Soc.* **79,** 3729 (1957).
11. TIMASHEFF, S. N., STURTEVANT, J. M., AND BIER, M., *Arch. Biochem. Biophys.* **63,** 243 (1956).